METABOLIC FRACTIONATION OF CARBON ISOTOPES IN MARINE PLANKTON, PART I: TEMPERATURE AND RESPIRATION EXPERIMENTS*

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ABSTRACT

The fractionation of stable carbon isotopes by marine phytoplankton cultures growing under defined environmental conditions has been determined. Skeletonema costatum and Cyclotella nana experiments indicate that an increase in temperature from 10° to 30°C causes a lowering of the δC^{13} difference between cells and HCO_3^- of ~ 0.35 ‰ per degree C when air (0.03 percent CO_2) is bubbled through the growing culture. No temperature effects are noticeable when 5 percent CO_2 in air is administered at a rapid flow rate resulting in a pH of 5.8 nor when $NaHCO_3$ is added in excess.

The data support the inference that both algae directly utilize molecular CO_2 rather than carbonic acid, bicarbonate, or carbonate. When air CO_2 is provided to an algal culture, there is an increasing drain on the molecular CO_2 supply as the culture becomes more dense, as the pH rises, and with increase in temperature. Consequently, the equilibrium between the molecular CO_2 and the pool of ionized species is broken down; C^{13}-enriched molecular CO_2 is released from the bicarbonate and is subsequently taken up by the plants. On the basis of this scheme, there is no need to postulate temperature-sensitive enzymatic reactions to explain the observed isotope fractionations

with change in water temperature.

When molecular CO_2 is sufficiently abundant, there is a constant fractionation of about 19 ‰ between CO_2 and the cells at all temperature levels (10° - 30°C).

Skeletonema costatum and Dunaliella tertiolecta cultures kept in darkness for 12 days show a depletion in C^{13} by as much as 5 ‰ relative to the C^{13}-content at the beginning of the period of dark respiration. Thus isotope fractionations in living material occurs in both synthetic and degenerative processes.

INTRODUCTION

The published record shows an 11 percent variation in the ratio of C^{13} to C^{12} in the organic carbon composition of natural materials. This wide range combined with the precision of carbon isotope analysis of ± 0.01 percent may explain the great interest and activity presently displayed in this area of research.

Stable carbon isotopes are widely used for the interpretation of specific biological and geological problems. For example, they have served as natural tracers in investigations concerning the "biosynthetic crossroads" of carbon during photosynthesis, and in studies of the origin of petroleum, coal, and kerogen. For a recent review on this subject, see Degens (1967). The complexities involved in the photosynthetic fixation of carbon are shown in work by Park and Epstein (1961) on tomato plants and by Abelson and Hoering (1961) on Chlorella cultures. Of similar interest are the studies by Kaplan and Rittenberg (1964) on carbon isotope fractionation occurring during the metabolism of lactate by the sulfate reducing bacterium Desulfovibrio desulfuricans. We also draw attention to Rosenfeld and Silverman's (1959) discovery that during fermentation by methane producing bacteria a methane is obtained enriched by as much as 90 ‰ in C^{12} relative to the methanol starting material. These authors also made the interesting observation that a rise in temperature increased methane production and δC^{13} in the methane liberated.

In this context, work by Sackett, Eckelmann, Bender, and Bé (1965) on the apparent temperature dependence of carbon isotope composition in marine plankton and sediments is of special interest. These authors found that marine plankton collected in South Atlantic waters near 0°C was depleted by several ‰ in C^{13} relative to samples from tropical regions. This isotope difference was also reflected in sediments taken at the respective sites. Aside from its biological significance, this observation has to be taken into account when interpreting the carbon isotope distribution in recent and ancient sediments.

Our work on the carbon isotope composition of marine plankton has similar objectives. . In the first of two articles we will be concerned with the effect of water temperature and length of respiration on the C^{13}/C^{12} ratio of laboratory-cultured marine phytoplankton. The second article (Degens, Behrendt, Gotthardt and Reppmann, 1967) chiefly deals with internal carbon isotope fractionation between the major biochemical constituents in plankton collected during the ANTON BRUUN Cruise 15 (April 13-26, 1966) in the South Pacific.

CULTURE METHODS

The neritic diatom Skeletonema costatum (WHOI clone "Skel"), the estuarine clone "3H" of the diatom Cyclotella nana, and the green flagellate Dunaliella tertiolecta (clone "Dun") were grown

in enriched sea water (half strength medium "f", Guillard and Ryther, 1962) under mixed "cool-white" and "natural" fluorescent lights (Sylvania) providing 5000 - 10,000 lux, in either a 14 hr. light, 10 hr. dark cycle, or in continuous light.

For study exclusively of the isotope fractionation that occurred during growth, and when air or 5 percent CO_2 provided the carbon, Cyclotella or Skeletonema cultures were grown in 1 - 2 l of medium in 2.4 l Fernbach flasks fitted with non-toxic silicone stoppers having tubes for aeration, venting, inoculation, and siphoning. When bicarbonate provided the carbon, the cultures were grown in 1 or 2 l glass-stoppered bottles, and sterile filtered $NaHCO_3$ was added to give a final concentration of inorganic carbon about ten times the 2mM found in natural sea water. (The algal crop is thus of the order of 1 percent of the carbon initially present.)

For study exclusively of the fractionation that occurred during respiration in prolonged darkness, Dunaliella cultures were grown in half liter lots in glass-stoppered bottles, with a total bicarbonate concentration eight times that of sea water. When growth was sufficiently dense, one flask was taken as the "time zero" sample, while the others were placed in darkness, to be removed one at each subsequent sampling time.

For a study of fractionation at different temperatures during both growth and subsequent dark respiration, Skeletonema cultures

were grown in 12 - 18 l batches in carboys fitted with the necessary tubes. After suitable cell densities were attained, the cultures were sampled and kept in darkness for the duration of the respiration study.

The cells were harvested by continuous or batchwise centrifugation and kept frozen at -20°C until required for analysis.

Initial and final 200 ml samples of algal medium were provided for determination of the isotope ratio in the total CO_2. Water samples were stored at 4°C with $HgCl_2$ added to prevent bacterial action on any dissolved organic matter.

MASS SPECTROMETER ANALYSIS

Organic carbon samples were converted to carbon dioxide and subsequently purified of any contaminating gas in the system following a procedure outlined by Craig (1953) and Sackett and Thompson (1963). This involves complete combustion of organic matter at about 900°C in the presence of copper oxide and oxygen, and the collection of the released CO_2 by liquid nitrogen traps. The carbonic acid species in water samples were released by acidification and were collected as CO_2 (Deuser and Degens, 1967).

The carbon isotope composition of the carbon dioxide, obtained either by the combustion of organic matter or the acidification of the aqueous media, was analyzed by a 6-in, 60° sector Nier-type mass spectrometer employing a double collecting system. Data are

reported as ‰ deviation relative to the PDB Chicago belemnite standard (Craig, 1953; 1957):

$$\delta\ C^{13} = (\frac{R}{R_s} - 1) \times 1000$$

$R = C^{13}/C^{12}$ ratio in sample

$R_s = C^{13}/C^{12}$ ratio in the standard

Appropriate correction factors described by Craig (1957) were applied.

When differences between two $\delta\ C^{13}$ values are of interest, the term $\Delta\ C^{13}$ is used.

RESULTS

The isotope data are summarized in Tables 1 and 2 and are grouped according to species, water temperature, and (in Table 2) according to days of respiration. Cyclotella and Skeletonema populations grown under approximately normal sea water conditions; i.e., pH 8.1 - 8.6, with CO_2 recharged from air, exhibited a similar isotope pattern; namely, an increase of temperature increased the C^{13} content of the cell carbon (Table 1). The relationships are illustrated in Figs. 1 and 2. It is noteworthy that the speed of aeration had a significant effect on the $\delta\ C^{13}$ of the cells.

When 5 percent CO_2 was supplied to the growing Cyclotella

population, the pH of the medium was lowered to 5.8 - 5.9. Under these conditions a change in water temperature from 10° to 30°C had very little effect on the isotope composition of the cell. Inasmuch as the 5 percent CO_2 supply was isotopically light with a δC^{13} of -26.1, the resulting plankton material exhibited highly negative C^{13} values. To facilitate comparison with the Cyclotella experiment done under normal sea water conditions, the fractionation difference between the CO_2 supply and the organic matter of the cells (ΔC^{13} cell-CO_2) has been calculated for each experimental condition (Table 1, last column; Fig. 2). The ΔC^{13} (cell-CO_2) values for the 5 percent CO_2 grown cells were identical to the ΔC^{13} (cell-HCO_3^-) for the Cyclotella grown at 10°C in aerated sea water. However, relative to the bicarbonate in the 5 percent CO_2 experiment, the cell fractionation increased to about 27 ‰ (ΔC^{13} (cell-HCO_3^-) column 6 of Table 1; Fig. 2).

A third experiment was performed under conditions of increased bicarbonate ion concentration (10 times that of normal sea water, achieved by adding $NaHCO_3$; pH 8.2). As was the case for the 5 percent CO_2 experiment, no temperature dependent isotope effects were noticeable. The fractionation between bicarbonate (δC^{13} -4.9 and plant cells δC^{13} -31) amounted to about -26 ‰ (Table 2; Fig. 1).

To test the effect of prolonged respiration on the isotope pattern of cell material, Dunaliella and Skeletonema populations were exposed for up to 19 days to darkness. Dunaliella showed a decrease

in $\delta\ C^{13}$ by about 5 ‰ in 5 days; from then on only a minor $\delta\ C^{13}$ decline was observable (Fig. 3). In case of Skeletonema, the changes were less pronounced and no systematic $\delta\ C^{13}$ trend could be recognized.

DISCUSSION

At present there is still uncertainty regarding the form in which carbon is taken up by marine plants during photosynthesis. The problem revolves around the possibility that molecular CO_2, bicarbonate, or some organic compounds may serve as carbon donor. The complexity of the subject is revealed in the work of Steemann Nielsen (1960, 1963, 1966), Watt and Paasche (1963) and Paasche (1964). The general consent is that molecular CO_2 is the principal form utilized directly during photosynthesis. The only marine species so far clearly shown to use bicarbonate is Coccolithus huxleyi, which lays down calcium carbonate. Fixation of CO_2 from dissolved organic materials such as carbamino carboxylic acids has been suggested; the data, however, are inconclusive. Before discussing the significance of the carbon isotope data, we will briefly consider some environmental aspects.

The relationships in the sea water carbonate system have been investigated by Saruhashi (1955). A recent review is presented by Skirrow (1965). In the context of the present work the effect of temperature and pH on the concentration and relative proportions

of the carbonic acid substances in sea water is of special interest. The solubility of carbon dioxide in sea water at 0°C is about 2.5 times greater than at a temperature of 30°C. Also, the P_{CO_2} of sea water is temperature dependent. In using data of Harvey (1955) the temperature coefficient for P_{CO_2} is about 1 percent per °C. This value corresponds to a change in CO_2 content of about 14 p.p.m. per °C. It is implied that increase in temperature will lower the total content of dissolved CO_2 in sea water, whereas the P_{CO_2} goes up. In using Harvey's data and assuming a constant pH, there is a net gain of concentration in molecular CO_2 in cold relative to warm waters.

The pH relationships in a sea water system are illustrated in Fig. 4. As the pH is lowered, the molecular CO_2 content increases.

Work by Deuser and Degens (1967) on the carbon isotope fractionation in the system CO_2(gas)-CO_2(aqueous)-HCO_3^-(aqueous) has shown that the carbon isotope fractionation between gaseous CO_2 and HCO_3^- decreases from 9.2 to 6.8 ‰ over the temperature range 0 to 30°C. This fractionation occurs in the hydration stage, not in the passage of atmospheric CO_2 through the air-water interface. Under isotopic equilibrium conditions, molecular CO_2 in sea water is virtually identical to atmospheric CO_2 whereas bicarbonate and molecular CO_2 should differ by about 7 to 9 ‰ in natural environments, depending on the temperature.

In light of these results, the carbon isotope data of this

study are easily interpretable and we refer to Fig. 1. The highest fractionation between bicarbonate and plant cells is achieved when CO_2 is most abundant (experiment: 5 percent CO_2). Next in line fall the experiments with a tenfold excess of HCO_3^- over normal sea water. The slight decrease in ΔC^{13} (cell-HCO_3^-) with increase in temperature in both experiments is consistent with the data of Deuser and Degens (1967) showing a change in the degree of fractionation in the same direction. The remaining three sets of experiments, i.e., under violent, intermediate and slow aeration conditions, and which may approach conditions normally found in the marine environment, reflect the decreasing availability of molecular CO_2 as the temperature increases and the air supply is reduced. The increasing drain on the molecular CO_2 supply breaks down the isotopic equilibrium between the molecular CO_2 and the pool of ionized species, and more heavy CO_2 enters the molecular CO_2 pool and is immediately taken up by the plants before equilibration is achieved. The internal temperature effect noticeable in the last three sets of experiments only reflects a change in the relative abundance of the forms of dissolved carbon and a difference in the growth rate of the organisms. For example, at 5°C the growth rate for Skeletonema and Cyclotella populations is about 5 times less than at 25°C, whereas the solubility of CO_2 is 2 times greater in the 5° relative to the 25°C water. The same isotope effects may also be achieved by changing the pH. As a rule, a

decrease in pH will increase the observed fractionation between the bicarbonate and the cell carbon. It appears, however, that at a certain point a minimum fractionation barrier will be reached. The Skeletonema experiment where air was bubbled only slowly through the medium, shows a $\Delta\ C^{13}$ (cell-HCO_3^-) of about -12.5 ‰ at 18° and at 28°C. In contrast, a maximum fractionation barrier at about the -28 ‰ level appears to be established in the Cyclotella experiment involving 5 percent CO_2. By calculating the $\delta\ C^{13}$ of the molecular CO_2 in equilibrium with the bicarbonate using the fractionation factors experimentally determined (Deuser and Degens, 1967), and calculating the $\Delta\ C^{13}$ (cell-CO_2) values for those experiments where molecular CO_2 was highly abundant, the fractionation amounts to about 19 ‰ for all temperature levels concerned.

In summary, Skeletonema and Cyclotella utilize exclusively molecular CO_2. If molecular CO_2 is highly abundant and in isotopic equilibrium with bicarbonate, the $\Delta\ C^{13}$ (cell-HCO_3^-) may be as high as -28 ‰. As the molecular CO_2 pool becomes effectively drained due to a number of environmental or biological circumstances (e.g., pH, temperature, rate of growth), the $\Delta\ C^{13}$ (cell-HCO_3^-) may be lowered to about -12 ‰. This wide range of isotope values that can be obtained from a single population just by varying certain environmental parameters may be quite helpful in future studies, when one intends to study phytoplankton populations in their

natural habitat. The data of Sackett, Eckelmann, Bender, and Bé (1965) who observed that plankton from arctic regions is depleted in C^{13} by about 6 ‰ ($\delta\ C^{13} \simeq -26$) relative to plankton collected in tropical waters, can now be interpreted in a reasonable fashion. A plankton sample collected in the Gulf Stream and analyzed by Craig (1953) gave a $\delta\ C^{13}$ value of about -12 ‰. This wide spread of about 15 ‰ between the various natural plankton samples so far examined is undoubtedly a function of the environmental circumstances under which the plants grew.

The respiration experiments for Dunaliella showed a decreasing $\delta\ C^{13}$ trend which may mean that C^{13}-enriched compounds are preferentially eliminated via respiratory carbon dioxide or organic excretion products. Consequently, C^{13}-depleted compounds will become concentrated. In view of the significant loss in carbon during respiration which amounts to 50 - 60 percent in 20 days (Hellebust and Terborgh, in press), such a drastic alteration is bound to leave an imprint on the carbon isotope composition of the organic residue. Less regular was the $\delta\ C^{13}$ pattern in the respiration experiments for Skeletonema. After an initial increase in $\delta\ C^{13}$ during the first day, $\delta\ C^{13}$ decreased and reached a plateau value in less than six days.

For comparative purposes, we include two isotope determinations on cultures of the marine chemoautotrophic bacterium Nitrosocystis

oceanus. This bacterium assimilates CO_2 via the reductive pentose phosphate cycle (Campbell, Hellebust and Watson, 1966). The observed carbon isotope fractionation between HCO_3^- and cell carbon was about twice that commonly observed in marine phytoplankton (Table 1). The pH at the start of Nitrosocystis growth is about 7.6, but this is rapidly lowered to pH 5.5 by oxidation of ammonia. Most of the dissolved carbon is present as molecular CO_2 under the latter condition of pH. Phytoplankton grown under such circumstances would have a ΔC^{13} (cell-CO_2) of about -19, rather than the -28 shown by the bacterium. While it is possible that the high fractionation of Nitrosocystis results from efficient fractionation in an enzymatic carboxylation at the ribulose -1, 5-diphosphate level, it seems more likely that it is due to the greater abundance ($\sim$ 25 percent) of C^{13}-depleted lipid compounds in Nitrosocystis. A carbon isotope study of Nitrosocystis is in progress (Watson, S. W., W. G. Deuser and E. T. Degens, unpublished).

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Table 1. The C^{13} content in molecular CO_2, HCO_3^- and cell carbon

(Relative to PDB_1 Standard)

Species	pH	Water Temp. °C	δC^{13} Molecular CO_2	δC^{13} HCO_3^-	δC^{13} Cell Carbon	ΔC^{13} Cell-HCO_3^-	ΔC^{13} Cell-CO_2
		10	(-5.7)*	+2.4	-16.4	-18.8	-10.7
Cyclotella	8.6	20	(-5.7)	+1.5	-15.5	-17.0	-19.8
(air)		30	(-6.1)	+0.7	-12.1	-12.8	- 6.0
		10	(-13.0)	-4.9	-31.1	-26.2	-18.1
Cyclotella	8.2	20	(-12.2)	-4.9	-30.9	-26.0	-18.7
(10 x excess HCO_3^-)		30	(-11.7)	-4.9	-30.5	-25.6	-18.8
		10	-26.1	-16.7	-44.7	-28.0	-18.6
Cyclotella	5.8	20	-26.1	-17.4	-45.0	-27.6	-18.9
(5% CO_2)		30	-26.1	-17.5	-44.5	-27.0	-18.4
		8	(-5.0)	+3.4	-13.4	-16.8	- 8.4
Skeletonema	8.6	18	(-5.0)	+2.6	- 9.7	-12.3	- 4.7
(slow aeration)		28	(-5.9)	+1.0	-11.5	-12.5	- 5.6
	8.0	8	(-7.0)	+1.3	-19.7	-21.0	-12.7
	8.2	18	(-6.2)	+1.2	-17.7	-18.9	-11.5
Skeletonema	8.2	18	(-7.6)	-0.2	-19.4	-19.2	-11.8
(violent aeration)	8.3	26	(-7.2)	-0.2	-17.3	-17.1	-11.1
	8.3	26	(-9.4)	-2.4	-19.4	-17.0	-11.0

Table 1 - Continued

Dunaliella		18	(-7.3)	+0.1	-16.2	-16.3	- 8.9
Nitrosocystis	5.5 - 7.6	20	(-7.3)	0.0	-35.9	-35.9	-28.6
(air)		20	(-7.3)	0.0	-35.4	-35.4	-28.1

* δC^{13} Values in parenthesis have been calculated from δC^{13} values in HCO_3^- column using equilibrium values determined by Deuser and Degens (1967).

TABLE 2

Variations in C^{13} Content in Marine Plankton as a Function of Length of Respiration

(relative to PDB_1 Standard)

Species	Length of Respiration (in days)	δC^{13} HCO_3^-	δC^{13} Cell Carbon	Temperature °C
Dunaliella	0	0.0	-16.2	18
	1	+0.3	-18.8	18
	5	+0.4	-20.9	18
	12	n.d.	-21.8	18
	19	n.d.	-21.7	18
Skeletonema	0	+3.4	-13.4	8
	1	n.d.	-10.9	8
	6	n.d.	-11.8	8
	12	n.d.	-11.8	8
Skeletonema	0	+2.6	- 9.7	18
	1	n.d.	- 9.2	18
	6	n.d.	-10.5	18
	12	n.d.	-10.4	18

FIGURE LEGENDS

Fig. 1. Diagram illustrating the influence of temperature and of the method of supplying carbon to growing algal cultures on the C^{13} enrichment of the algal cells relative to the bicarbonate of the medium. Data are given in Table 1.

Fig. 2. Diagram illustrating relationships between the C^{13} content of Cyclotella nana cells (circles), molecular CO_2 of the medium (squares) and bicarbonate of the medium (triangles) in three sets of experiments, in which air (upper set), excess bicarbonate (middle set) and 5% CO_2 (bottom set) served as source of carbon. Data are given in Table 1.

Fig. 3. Decline of C^{13} content of Dunaliella tertiolecta kept in continuous darkness. Data are given in Table 2.

Fig. 4. Distribution of forms of inorganic carbon in fresh and sea water, as percent of total present. "Dissolved CO_2" is the sum of the carbonic acid (H_2CO_3) and "molecular CO_2" present. Modified from Skirrow (1965, Fig. 4).

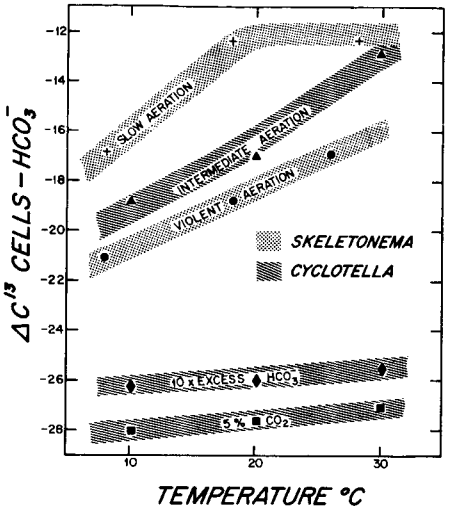

Figure 1

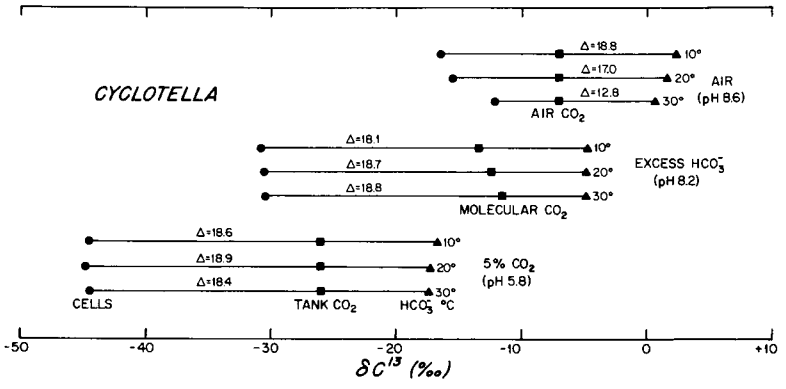

Figure 2

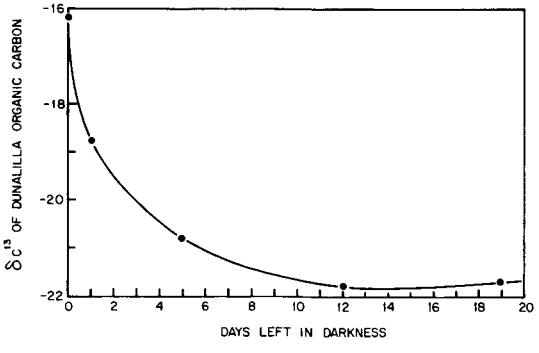

Figure 3

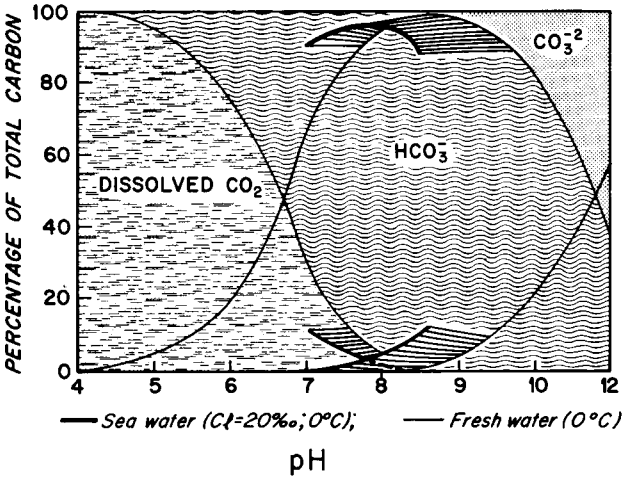

Figure 4